SOLUBILIZATION OF CHICK KIDNEY MICROSOMAL CYTOCHROME *P*-450

Electron paramagnetic resonance studies of ligand interactions

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1. Introduction

Cytochrome *P*-450 is the generic name given to the heme protein which exhibits a Soret $A_{450\ nm}$ max in its reduced carbon monoxide-complexed state. The cytochrome was first discovered as a CO-binding pigment in isolated mammalian liver microsomes [1,2]. Since then a host of similar heme proteins have been described in adrenal and kidney mitochondria [3,4] and in bacteria [5]. The cytochromes are predominantly involved in drug, steroid and hydrocarbon metabolism.

The characteristic visible absorption bands of cytochrome *P*-450 from all sources may change upon the binding of specific substances to the cytochrome. In general, substrates and certain other molecules induce a shift in the spectrum of the cytochrome to the blue (type I) whereas aromatic primary amines and aromatic N-heterocyclic compounds shift the spectrum to the red (type II). The type I spectral change is characterized by an A_{390} max and A_{420} min. The type II change exhibits an A_{390} min and A_{420} max These ligand-induced optical properties may be due to changes in the spin state of the heme, or to minor perturbations in the heme environment.

Ligand-induced spectral properties of cytochrome *P*-450 have also been studied with electron paramagnetic resonance spectroscopy (EPR). The oxidized cytochrome is paramagnetic with *g* values near 2.42, 2.26 and 1.91 (low spin), and near 8, 4 and 1.8 (high spin) [6]. Spin state interconversions and spectral modifications of the low and high spin resonances of cytochrome *P*-450 have been described [6], but many interactions of the cytochrome with ligands do not give rise to large alterations in spin state ratios.

The involvement of a cytochrome *P*-450-dependent monooxygenase system in the kidney mitochondrial 1α-hydroxylation of 25-hydroxyvitamin D_3 (25-OH-D_3) to yield 1,25-$(OH)_2D_3$ has been described [4,7]. This product is currently regarded as the physiologically active metabolite associated with the movement of calcium and phosphate ions in the intestine and skeleton. More recent work [8,9] has demonstrated that a kidney microsomal cytochrome *P*-450 system is likewise associated with the metabolism of 25-OH-D_3. Both of these cytochrome *P*-450-catalyzed monooxygenase reactions occur in the vitamin D_3-deficient chick. The microsomal reaction could not be demonstrated to occur in the vitamin D_3-supplemented chick

Abbreviations: 25-OH-D_3, 25-hydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1,25-hydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; DTT, 1,4-dithiothreitol; METY, metyrapone; EtOH, ethanol

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[8]. However, in the vitamin-supplemented chicks, 25-OH-D_3 is metabolized by the kidney mitochondria to 24,25-$(OH)_2D_3$ whose exact biological functions remain elusive.

As a result of the kidney's central role in the production of the biologically functional metabolites of vitamin D_3, a thorough examination of the properties of the kidney cytochrome *P*-450 systems is necessary. Results of such examinations may serve to define the intricacies of the regulatory mechanisms of vitamin D_3 metabolism at the molecular level. We here report, therefore, on the application of EPR and optical spectroscopy to the study of the effects of specific ligands on the magnetic and optical properties of cytochrome *P*-450 in the chick kidney microsomes.

2. Materials and methods

2.1. *Animals*

One-day old white Leghorn cockerel chicks (Northern Hatcheries, Beaver Dam, WI) were maintained on a vitamin D-deficient rachitogenic test diet (US Biochemicals, Cleveland, OH) for 4 weeks before use. Alternatively, to obtain vitamin D-supplemented animals, a similar group of one-day old chicks were housed separately and maintained on a normal laying mash (Waldschmidt and Sons, Menomonee Falls, WI). Each bird was also given orally a vitamin D_3 supplement 5 days a week for 2 weeks at a level of 10 units (250 ng) vitamin D_3 in 50 μl Wesson oil. The chicks were then allowed to rest for 1 week prior to killing by cervical dislocation.

2.2. *Preparation of microsomes*

The kidneys, either from D-deficient or D-supplemented chicks, were removed and rinsed with chilled buffer (0.25 M sucrose/15 mM Tris–acetate, pH 7.4, 1 mM DTT and 0.1 mM EDTA). After removing the adhering connective tissues, the cleaned kidneys were homogenized in 4 vol. (w/v) of the buffer using an ice-cold Potter-Elvehjem homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 500 × *g* (700 rev/min in a Sorvall SS-34 rotor) at 4°C for 10 min. The postnuclear supernatant was then centrifuged at 20 000 rev/min in a Beckman model L preparative ultracentrifuge at 4°C for 30 min using a 30 rotor. The pellet was discarded. This centrifugation was repeated and the pellet discarded again. The resulting supernatant was then centrifuged at 30 000 rev/min in the model L ultracentrifuge at 4°C for 60 min. The supernatant was discarded. The red microsomal pellet was suspended in fresh buffer with gentle homogenization to a protein concentration of 8 mg/ml as determined by the method [10].

2.3. *Solubilization of the microsomal cytochrome P-450*

Frozen (–80°C) microsomes as prepared above were thawed and centrifuged at 30 000 rev/min in the model L ultracentrifuge at 4°C for 60 min. The supernatant was discarded. The pellet was suspended in 10% glycerol, 0.1 M potassium phosphate buffer, pH 7.4, 1 mM DTT and 0.1 mM EDTA. The protein concentration was adjusted to 8 mg/ml suspension as determined by the method [10]. A 10% Lubrol-WX solution in water (w/v) (Arnold, Hoffman and Co., Providence, RI) was added to the microsomal suspension at the level of 1.5 mg Lubrol/mg protein. The mixture was allowed to stand on ice for 30 min with occasional agitation. The mixture was then centrifuged at 30 000 rev/min in the model L ultracentrifuge at 4°C for 60 min. The pellet was discarded. The supernatant containing approx. 0.12 nmol cytochrome *P*-450/mg protein (from vitamin D-supplemented) or 0.27 nmol/mg protein (from vitamin D-deficient) was concentrated to about 30 mg protein/ml using an Amicon ultrafiltration cell fitted with a PM-30 membrane. The cytochrome concentrations represent 50% recovery relative to intact microsomes.

2.4. *Analytical methods*

The concentrations of the chick kidney microsomal cytochrome *P*-450 were determined from the reduced-CO minus the oxidized-CO difference spectra. The concentration of the pigment in the normal rat liver microsome was determined from the reduced-CO minus the reduced difference spectrum. A value of 91 cm^{-1} mM^{-1} was used for the molar extinction increment between the wavelengths 450 nm and 490 nm [11]. The CO used was scrubbed free from traces of oxygen by bubbling through a 10% pyrogallol in 10% KOH solution. All optical measurements were performed at room temperature with an AMINCO DW-2 UV/VIS spectrophotometer. EPR measurements were performed using a Varian E-9

X-band spectrometer system equipped with 100 kHz field modulation. First derivative spectra were generally obtained at 103°K by blowing cold nitrogen over the sample. For temperatures below 77°K an Air Products LTD-3-110 helium transfer system was employed. Spin concentrations were estimated relative to a rat liver microsomal preparation containing 6.6 μM cytochrome *P*-450, 93% of which was taken to be in the low spin form [12]. Absorbance measurements of the chick kidney cytochrome *P*-450 gave concentrations that were slightly lower than the spin concentrations.

3. Results

3.1. *EPR measurements*

Signals characteristic of low spin cytochrome *P*-450 were detected at 103°K from both intact microsomes and solubilized *P*-450 preparations. The spin concentrations in these samples were estimated to be in the range of 1.1–6 μM. Examination of the spectra at temperatures down to 8°K did not reveal detectable amounts (μM range) of the high spin cytochrome. Small quantities of high spin ferric non-heme iron (*g* 4.3) [13] were present in all the samples. The *g* values of the intact microsomal *P*-450 cytochromes (D_3-deficient and D_3-supplemented) were close to those measured for the respective solubilized heme proteins as shown in table 1. Intact microsomes further contained some high spin ferric heme (*g* 6).

The spectra and the EPR parameters of the low spin solubilized cytochrome *P*-450 are given in fig.1 and 2 and in table 1. Small differences in the *g* values (2.422 cf. 2.415) of the low field extremum were found in the spectra of the *P*-450 cytochromes solubilized from D_3-deficient and D_3-supplemented chick kidney microsomes. Addition of metyrapone (75 μM) effected a dramatic change in the D_3-deficient cytochrome spectrum (g_1 2.449 cf. 2.422; g_2 2.261 cf. 2.255) while it produced no significant change in that of the D_3-supplemented pigment. This change with the D_3-deficient *P*-450 is similar to those observed with *P*-450 cytochromes of rat liver microsomes [14] and bovine adrenal mitochondria [15]. Ethanol (0.31 M) and 25-OH-D_3 (75 μM) produced smaller changes. None of the reagents employed significantly changed either the linewidth or the spin concentration.

3.2. *Optical measurements*

To measure the affinity of cytochrome *P*-450 for METY or 25-OH-D_3, the spectral dissociation con-

Table 1
EPR parameters and spin concentrations for low spin cytochrome *P*-450

Cytochrome *P*-450	g_1	g_2	g_3	[*P*-450] × 10^6 M
In the intact microsomes				
D_3-deficient	2.422	2.255	1.924	4.0
D_3-supplemented	2.417	2.255	1.926	1.1
Solubilized from microsomes				
D_3-deficient	2.422	2.255	1.924	6.1
+ METY (75 μM)	2.449	2.261	1.922	6.1
+ EtOH (0.31 M)	2.412	2.251	1.922	6.1
+ 25-OH-D_3 (75 μM)[a]	2.412	2.253	1.922	6.1
D_3-supplemented	2.415	2.255	1.925	2.7
+ METY (75 μM)	2.415	2.258	1.928	2.7
+ EtOH (0.31 M)	2.406	2.254	1.926	2.7
+ 25-OH-D_3 (75 μM)[a]	2.404	2.252	1.925	2.7

[a] Calculated from the ultraviolet spectrum of stock solutions in 95% EtOH using a value for ϵ_{264} of 18 cm^{-1} mM^{-1}. The substrate, 25-OH-D_3, was added in 95% EtOH which also gave a final EtOH concentration of 0.31 M

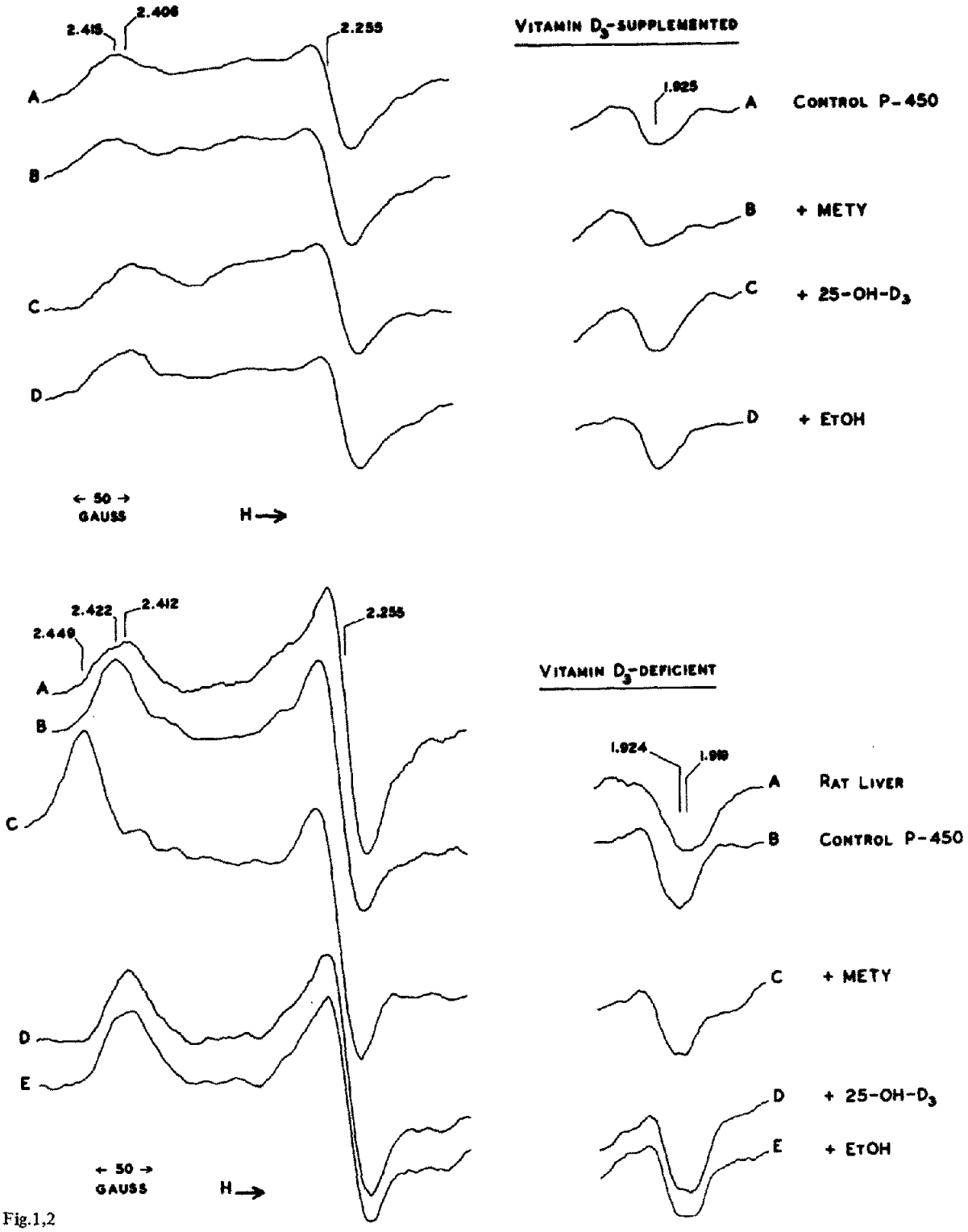
2.416
2.406
2.255
A
B
C
D
← 50 →
GAUSS
H
VITAMIN D3-SUPPLEMENTED
1.925
A
CONTROL P-450
B
+ METY
C
+ 25-OH-D3
D
+ ETOH
2.422
2.412
2.449
2.255
A
B
C
D
E
← 50 →
GAUSS
H
VITAMIN D3-DEFICIENT
1.924
1.919
A
RAT LIVER
B
CONTROL P-450
C
+ METY
D
+ 25-OH-D3
E
+ ETOH

Fig.1,2

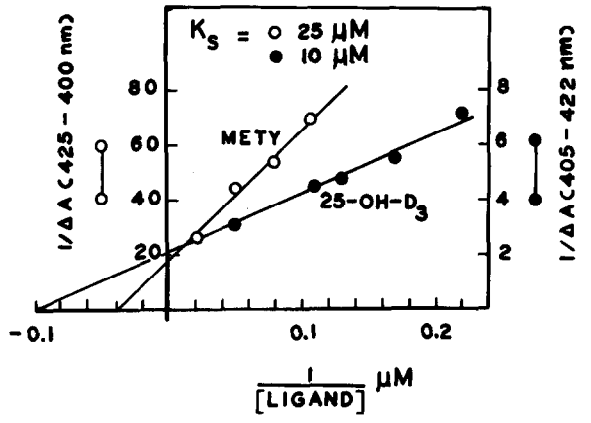

Fig.3. Double reciprocal plot for the determination of spectral dissociation constants. The concentration of the solubilized microsomal cytochrome *P*-450 used was 2.7 μM for the D_3-deficient state (○) and 1.8 μM for either D_3-deficient or D_3-supplemented state (●). Samples contained approx. 30 mg protein/ml in 10% glycerol, 0.1 M potassium phosphate, pH 7.4, 1 mM DTT and 0.1 mM EDTA. Dual wavelength mode of the DW-2 spectrophotometer was used equipped with a single cuvette of 1 cm light path and 2 mm cell width containing 0.6 ml sample. Full-scale deflection was set at 0.02 A. The wavelength pairs were chosen on the basis of reported data [4,9]. Titrations were performed at room temperature by adding 0.2 μl aliquots of either 7.5 mM METY or 1.5 μM 25-OH-D_3. The difference in absorbance was taken as a measure of ligand–*P*-450 complex formation.

stants, K_s, were determined according to the method in [16] (fig.3). The K_s value (25 μM) determined for METY with the solubilized heme protein from D_3-deficient chicks is similar to that (33 μM) reported [4] for the intact microsomes of analogous animals. However, using solubilized cytochrome *P*-450 from D_3-supplemented chicks, no such interaction was detected. In contrast, 25-OH-D_3 interacted equally well with either pigment. A K_s value for either interaction was determined to be 10 μM (a K_m value of 2.5 μM was reported [9] for the in vitro metabolism of 25-OH-D_3 by the intact D_3-deficient microsomes).

4. Discussion

It has been argued [17] that the substrate binding site (site I) on *P*-450 cytochromes differs from that of O_2, CO and inhibitors (site II). The data for the interactions of 25-OH-D_3 and METY with the solubilized pigments from either D_3-deficient or D_3-supplemented chick kidney microsomes indeed suggest that the METY binding site differs from that of the substrate, 25-OH-D_3. In view of this, an endogenous ligand bound to site II is perhaps responsible for the lack of an effect of METY on the EPR spectrum of the D_3-supplemented cytochrome. However, it is also possible that binding of endogenous ligands in the proximity of site II can render, via conformational effects, the heme inaccessible for interaction with METY. In either case, it appears likely that this endogenous ligand may be responsible for the lack of the in vitro metabolism of 25-OH-D_3 in the presence of the D_3-supplemented microsomes [8].

The intricacies of the regulation of vitamin D metabolism have been explored in several laboratories. It is known, for instance, that under certain physiological circumstances the principal metabolite produce from 25-OH-D_3 by the kidney is 1,25-$(OH)_2D_3$ and that synthesis of this product is tightly regulated by serum calcium [18,19] and phosphate [20] concentrations. However, the precise molecular mechanisms responsible for this regulation remain obscure. In recent work [9] we described a cytochrome *P*-450-dependent metabolic activity in kidney microsomes which appears to be associated with the metabolism of 25-OH-D_3. This activity is detectable only in vitamin D-deficiency [9] and its in vivo significance is not yet clear. However, the present report demonstrates that binding of 25-OH-D_3 to the *P*-450 cytochromes is independent of the vitamin D status of the animal. The inability of D_3-supplemented chick kidney microsomes to metabolize 25-OH-D_3 in vitro can now be attributed (at least in part) to the non-availability of the heme moiety of cytochrome

Fig.1,2. Effect of metyrapone (75 μM), 25-OH-D_3 (75 μM), and ethanol (0.31 M) on the EPR spectra of solubilized cytochrome *P*-450 from the kidney microsomes of D_3-deficient (fig.1) or D_3-supplemented (fig.2) chicks. Samples contained approx. 30 mg protein/ml in 10% glycerol, 0.1 M potassium phosphate, pH 7.4, 1 mM DTT and 0.1 mM EDTA. The cytochrome *P*-450 concentractions as determined from difference spectra were 4.2 μM for the D_3-deficient and 1.8 μM for the D_3-supplemented. EPR conditions were: microwave power, 100 mW; modulation amplitude 25 G; time constant 1.0 s; scan rate 250 G/min; temp. 103°K.

P-450 in the catalytic event. The precise biochemical relationship of this phenomenon to the overall regulation of vitamin D metabolism remains to be elucidated.

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